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"Commercial exploration of Insect Proteases"

By

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Synopsis

Introduction

Enzymes catalyze most of the biological reactions which serve basis for metabolic activities in all living organisms. It is not over claiming to say that 'any biological reaction can't possible without the involvement of any of the enzymes. Enzyme focused research is of importance not only to biological community but also to process designers/engineers, chemical engineers and researchers working in other scientific fields. Enzymes being proteins possess properties such as specificity towards the reactions they catalyze and the substrates on which they act upon. Apart from basic approach, enzymes are widely studied from an industrial point of view. From an industrial standpoint, only a limited number of enzymes are commercially available and few of them have found applications in large quantities. More than 75% of industrial enzymes are hydrolyses (Rao et al., 1998). They have variety of applications in various industries, particularly in detergent, leather, pharmaceutical, silver recovery, textile, tannery industries, food bioremediation, biosynthesis, and biotransformation (Shanmughapriya et al., 2008, Gupta et al., 2002, and Jellouli et al., 2009). With advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields such as clinical, medicinal and analytical chemistry (Joo et al., 2005).

Proteases occur naturally in all organisms. These enzymes are involved in a large number of physiological reactions from simple digestion of food proteins to highly-regulated cascades (the blood-clotting cascade, the complement system, apoptosis pathways, octadecanoid pathway etc.). Proteases can either break specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis). The activity of a particular protease is depends upon the function of the protein. The proteolytic activity can be a destructive change, abolishing a protein's function or digesting it to its principal components; it can be an activation of a function, or it can be a signal in a signaling pathway. Proteases are divided into four major groups according to the nature of their catalytic active site and conditions of action: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. Attachment of a protease to a certain group depends on the structure of catalytic site and the amino acid (as one of the constituents) essential for its activity. Proteases determine the lifetime of proteins which are playing important physiological role like

hormones, antibodies, or other enzymes. This is one of the fastest "switching on" and "switching off" regulatory mechanisms in the physiology of an organism. By complex cooperative action the proteases may proceed as cascade reactions, which result in rapid and efficient amplification of an organism's response to a physiological signal.

Proteases are among the oldest and most diverse families of enzymes known and they are involved in every aspect of organism's function. They constitute a very large and complex group of hydrolytic enzymes that degrade proteins into small peptides and amino acids. Proteases catalyze the addition of water across amide (and ester) bonds to cleave using a reaction involving nucleophilic attack on the carbonyl carbon of the scissile bond. They differ widely in their properties such as substrate specificity, active site and catalytic mechanism and possess different profiles for mechanical stress, chemical environment, pH and temperature for stability and activity. Because of their broad substrate specificity, proteases have a wide range of applications. In recent years a number of studies have been conducted to characterize protease from different microorganisms including bacteria, moulds, yeasts and also from mammalian tissues (Priest 1977). Bacteria are the most important protease producers with the genus *Bacillus* being the most prominent source, because of their capacity to produce large amounts of protease having significant proteolytic activity and stability at high pH and temperature. Microbial proteases constitute approximately 40% of the total worldwide production (Godfrey et al., 1996). Till date the major source to obtain industrially important protease is from different microorganism such as bacteria and fungi. In general most of the alkaline proteases are especially derived from *Bacillus* species (Maurer, 2004). Alkaline proteases from high yielding bacterial strains have been extensively studied. However, most of the alkaline proteases applied for industrial purpose have some limitations. First many of the available alkaline proteases exhibited low activity and stability towards anionic surfactants like Sodium dodecyl sulphate (SDS) and oxidants such as bleach and H_2O_2, which are common ingredients in modern bleach, based detergent formulations. Secondly around 30 to 40% of the production cost of industrial enzymes is estimated to be accounted for the cost of the proteases growth medium (Joo et al., 2003). The industrial demand of highly active preparations of proteolytic enzymes with appropriate specificity and stability towards alkaline pH, high temperature, surfactant and oxidizing agents attributed to stimulate the search for new enzyme

source form nature which has natural potency to exhibit activity and stability and less cost estimated for their production.

Thus considering the limitation of currently used microbial proteases in the detergent industries many researchers attracted towards alternative source of proteases. The use of insect midgut proteases is seems to be underestimated by the researchers in this field. The digestive enzymes of the insects are of interest both as a target for insect control and also because of their unusual ability to function in alkaline microenvironment in the gut pH 10.0–12.0 (Christeller et al., 1992, Applebaum, 1985). This observation substantiates that proteases from the insect guts are finely designed to work optimally in alkaline conditions of the guts (Teera, 1990). It is well documented that protein digestion in the insect larvae is mediated by the concerted action of digestive enzymes in the larval guts, particularly trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), which are the primary digestive proteases in most insect groups (Terra, 1996). Since proteolysis is an essential part of food digestion in insects, studies on insect proteases are important. Insect proteases may be used in several industrial processes since they have the important characteristics such as high temperature and alkaline pH optimum. Previously, it is showed that crude gut extract of *P. americana* contains several trypsin and chymotrypsin like proteases (Hivrale et al., 2005). Thus, it is of great importance to further investigate the industrial potential of the *P. americana* gut proteases. Present work was undertaken to find out the important chemostable proteases from *P. americana* which can act at highly alkaline pH and high temperatures and can be used for industrial proteases.

Objectives

1] Screening of detergent stable proteases from *P. americana*.

2] Identification, isolation and purification of individual protease.

3] Biochemical, molecular characterization and chemostability of purified proteases.

Methodology

Since cockroach can live in any adverse and temperate climates and on variable foods throughout the world, it must contain a bank of enzymes with different properties which give strength to cockroach to live in such environments. Considering this fact we have stated to work on the identification and characterization of *P. americana* midgut proteases that can have several industrially important properties.

Preparation of ceacea extract

Cockroaches were collected from grain godowns of Aurangabad, Maharashtra and immobilized by keeping at -20 ºC for 2 h and dissected mid ventrally. Ceacea were removed and homogenized with pre-chilled mortar and pestle in 1:6 (w/v) volumes of cold 0.1 M Tris-HCl pH 8.0. Collect the midgut ceacea extract as proteases source. Protease activities were quantified by proteases assay and gel X- ray film contact print method.

Total proteolytic activity assay

Total proteolytic activity was determined by casinolytic assay. Different protein concentrations from *P. americana* ceacea extract were added into 2 ml of 0.5% casein and the mixture was incubated for 20 min at 40 ºC. After 20 min the reaction was terminated by adding 5% trichloro acetic acid (TCA). The absorbance of TCA soluble peptidase was measured at 280 nm.

Screening and identification of detergent and chemostable protease

For isolation of desired protease, crude ceacea extract of *P. americana* were loaded on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protease stability towards detergent and various organic solvents and substrate specificity were checked by using specific chromogenic substrates namely BApNA, SAAPF*p*NA and L*p*NA. Among the studied proteases we have selected highest activity and detergent stable proteases for further process of purification and biochemical characterization.

Purification of selected proteases

Acetone precipitation

To the supernatant (50 ml) equal amount of chilled acetone was added and kept overnight at -20 ºC. Next day the precipitate was collected and centrifuged at 10,000 rpm for 20 min at 4 ºC. Acetone was removed by air drying and precipitate was collected. The precipitate was dissolved in minimum amount of 0.1 M Tri-HCl buffer pH 8.0 and centrifuged at 10,000 rpm for 20 min and the supernatant was collected.

Gel filtration chromatography

Sephadex G-100 (5 gm) gel was added in 0.1 M Tris-HCl (pH 8.0) and allowed to swell it for overnight and column was packed. The column was equilibrated with 0.1 M Tris-HCl buffer pH 8.0. Total 5 ml of acetone precipitate was loaded onto a Sephadex G-100 column (1.3 X 65 cm). The column was eluted with Tris-HCl buffer (pH 8.0) and fractions of 1 ml were collected at a flow rate of 1 ml/min. Protein concentration in each fraction was determined by Lowry method using bovine serum albumin as standard.

Molecular mass determination

Molecular mass of the purified enzymes was determined on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Purified protein was loaded on 12% SDS-PAGE with standard molecular mass markers and electrophoresis was carried out at a constant current of 30 mA. After electrophoresis the gel was stained with Coomasie Brilliant Blue R-250 (CBB R-250) and distained to visualize protein bands.

Biochemical characterization of purified proteases

Substrate specificity of purified proteases

The substrate specificity of the purified enzyme was examined using synthetic substrates such as BA*p*NA, SAAPF*p*NA and L*p*NA. Stock solutions (10 mM) of each substrate were prepared in dimethyl sulphoxide (DMSO). The reaction mixture was prepared by preincubating the purified enzyme with 0.1 M Tris-HCl (pH 9.0) for 10 min at 40 °C. The reaction was started by adding the reaction mixture in each substrate. Absorbance was measured at 410 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of p-NA/ min at 40 °C.

Effect of pH, Temperature and its stability on purified proteases

The activity of purified enzymes in various buffers were evaluated by adding 20 µL purified enzyme in pre-incubated 1 mL buffer of pH ranging from pH 3.0–12.0 (pH 3.0–12.0). The reaction was carried out at 37 °C and terminated after 20 min by adding TCA. The absorbance of TCA soluble peptidase was measured at 280 nm. The stability of purified protease at different temperatures (10–100 °C) was evaluated by adding 20 µL purified enzyme in substrate. The reaction was carried out at different preset temperatures for 20 min. After 20 min the reaction was terminated by adding TCA and subsequent hydrolysis of substrate was monitored at 280 nm. To examine the temperature stability, the purified enzyme was incubated at different temperature ranges from 50 to 70 ºC for 1 h and the residual enzyme activity was measured under standard assay conditions.

Effects of inhibitors and metal ions on purified proteases activity

The effect of enzyme inhibitors on purified proteases activity was studied using PMSF (1 mM), EDTA (5 mM), DTNB (5 mM) and β-mercaptoethanol (5 mM). The reaction mixture was prepared by pre-incubating the purified enzyme with inhibitors for 10 min at 40 ºC. The reaction was terminated after 30 min by adding TCA. The remaining protease activity was determined under standard assay conditions. The effects of monovalent (Na^+ and K^+) and divalent (Cd^{2+} , Zn^{2+}, Cu^{2+} , Ba^{2+} and Hg^{2+}) metal ions on purified protease activity at a concentration of 5, 10 and 15 mM were investigated

Effect of surfactants and oxidizing agents on purified proteases activity

The effect of surfactants (Tween 20, Tween-80, Triton X-100 and SDS) and oxidizing agents (H_2O_2 and bleach) wear studied the reaction was started by adding the reaction mixture and incubated for 1 h at 40 ºC. After 1 h incubation the reaction was terminated by adding TCA.

Stability of purified proteases towards organic solvents and commercial detergents

The effect of various organic solvents such as acetone, xylene, benzene, propanol, DMSO, toluene, ethanol, butanol and hexane at 25 % concentration on purified proteases activity were studied. Stability of purified proteases was checked against several commercial solid detergents such as ariel, tide, surf, wheel, henko, and rin. Detergent solutions were prepared to get final

concentration 1, 10 and 100 mg/ml of the each detergent. Prior to assay prepared detergent solutions were pre heated at 100 ºC for 1 h to destroy the endogenous protease activity present in the commercial detergent. The reaction was started by adding the reaction mixture and incubated for 1 h. After 1 h incubation the reaction was stopped by adding TCA. Residual enzyme activity was measured.

Results

Screening and identification of detergent and chemostable proteases

Cockroach crude midgut ceacea extract showed total 11 proteases activity isoforms on 12 % SDS PAGE. All these isoforms showed activity at alkaline pH and temperature ranging from 7 to 9 and 50 to 70 ºC respectively. All isoforms showed stability at alkaline pH and high temperature. Proteases were showed resistance towards surfactant, oxidizing agents, metal ions, organic solvent and detergents. Among these isoforms we have selected two isoforms which showed highest protease activity and stability towards various surfactants, oxidizing agents, heavy metal ions, organic solvents and commercial detergents at various concentrations. On the basis of substrate specificity we found that these isoforms was trypsin and chymotrypsin like serine alkaline proteases.

Biochemical and molecular characterization of purified trypsin

Trypsin like enzyme from *P. americana* was partially purified by acetone precipitation and gel filtration chromatography. The enzyme was purified near to homogeneity using acetone precipitation and Sephadex G-100 gel filtration chromatography. Specific activity of purified enzyme was increased up to 4.2 fold with 19% recovery. Molecular mass of the purified enzyme was found to be ~ 27.8 kDa on SDS PAGE. The synthetic substrate and inhibitor study showed that enzyme had maximum specificity towards BA*p*NA and its activity was completely inhibited by PMSF, so enzyme was characterized as serine protease belongs to trypsin family. The enzyme was characterized as serine protease since it was completely inhibited by PMSF. The enzyme was highly active in the pH range of 7.0 –12.0 with an optimum at pH 8.0, It showed stability at a pH range between 7.0 to 12.0 maintaining 90% of its original activity after 1 h incubation at 40 ºC. The enzyme was active at temperature from 50 to 70 ºC with an optimum at 60 ºC, thermal stability profile showed that the enzyme is fully active for at least

100 min between the temperature range 50 to 70 ºC. The effect of various metal ions showed that at a concentration of 5 mM enzyme retained almost 90% of its activity in presence of Ca^{2+}, Zn^{2+}, Ba^{2+}, Hg^{2+}, Cu^{2+}, Na^{+}, and K^{+}. The enzyme activity was slightly decreased by each metal ion at 10 mM concentration, while about 20% inhibition was observed at 15 mM concentration of each metal ion. Effect of various surfactants on enzyme activity showed that 93, 63, and 95% of its activity in presence of 1% Tween-20, Tween-80 and Triton X-100 respectively. The enzyme retained almost 60% of its activity in presence of these three nonionic surfactants at a concentration of 5 and 10% respectively. Whereas in oxidizing agents enzyme able to retain its 60% activity in the presence of 10% SDS. Nearly 14% enzyme activity was inhibited by H_2O_2 at the concentration of 1%, however at 5 and 10% concentration it inhibited around 37 and 36% activity respectively. Enzyme retained 80% of its activity in presence of 1% bleach whereas about 34 and 40% activity was inhibited by bleach at 5 and 10% concentration respectively.

For chemostability the effect of various organic solvents on protease activity was checked. We found that enzyme activity was increased up to 5 to 10% in presence of acetone, butanol, and hexane. Around 15% activities were inhibited by propanol, DMSO and toluene. The enzyme retained its 100% activity in presence of xylene, benzene and ethanol. Stability of enzyme towards the commercial detergents showed that the enzyme retained its 90% activity in presence of tide, wheel, surf and rin, whereas enzyme showed 80% of its activity in presence of aerial and henko respectively. Similar results were observed at each concentration of detergents.

Biochemical and molecular characterization of purified chymotrypsin

Chymotrypsin like serine alkaline protease from *P. americana* midgut ceacea was purified by acetone precipitation and gel filtration chromatography. The enzyme was purified up to 3.3 fold with 48% recovery. For molecular mass determination, the purified fraction was loaded on 12% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The enzyme was appeared as single band on 12% SDS-PAGE. The molecular mass of the purified enzyme was estimated to be ~ 63 kDa. Purified enzyme showed maximum specificity towards SAAPF*p*NA thus the enzyme can be considered as chymotrypsin like serine protease. Enzyme activity was completely inhibited by both chymotrypsin specific inhibitor chymostatin and serine protease inhibitor PMSF. Observed results indicate that the purified enzyme was serine-protease and belongs to the chymotrypsin family. The purified was found to be active at broad range of pH

from pH 6.0 to 12.0. The optimum pH for protease activity was around pH 9.0. The enzyme showed strong stability from pH 6.0 to 12.0 up to 1 h at 40 ºC. The relative activity at pH 12.0 was 85%.The optimum temperature for enzyme was found at 50 ºC. Enzyme activity gradually declined at temperature beyond 70 ºC. Thermal stability profile showed that enzyme was highly stable at temperature ranges from 50 to 70 ºC. The relative activities at 50, 60 and 70 ºC were about 80, 74 and 68% respectively. Effect of heavy metal ions on enzyme showed that at concentration of 1 mM enzyme retained about 85% of its activity in presence of Ag^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Na^{+} and K^{+}. Enzyme retained 106% its activity in presence of Mg^{2+}, whereas 50% enzyme activity was inhibited by Cd^{2+} metal ion. Enzyme showed 100% of its activity in presence of Hg^{2+}, Zn^{2+} and K^{+} whereas Cd^{2+} and Cu^{2+} inhibit 10% enzyme activity at 5 mM concentration. About 6% enzyme activity was inhibited at 10 mM concentration of all studied metal ions except Mg^{2+} it inhibit 15% activity. The effect of various anionic and nonionic surfactants showed at 0.1% concentration enzyme retained 86, 80 and 99% of its activity while at 1 and 10% it retained 75 and 70% activity in presence of non-ionic surfactants respectively. However, *P. americana* chymotrypsin was highly stable in anionic surfactant (SDS) and retained 84, 78, and 71% of its activity in presence of 0.1, 1 and 10% SDS respectively. Enzyme activity was also assessed in presence of oxidizing agents it retained 98, 82, and 64% of its activity at 0.1, 1 and 10% respectively in presence of H_2O_2. In bleach it retained its 96% activity at 0.1% concentration while at 1 and 10% concentration enzyme retained 104 and 107% of its activity.

Chemostability result showed that enzyme retained of its 105 and 107% activity in presence of propanol and ethanol. Enzyme retained its 100% activity in presence of xylene, hexane, benzene and toluene. About 40 and 50% protease activity was inhibited by butanol and acetone. Stability of enzyme with commercial detergent showed that at 0.1 % concentration enzyme retained about 95, 90, 75, 105, 96, 86, and 90% of its activity in presence of henko, rin, tide, ghadi, wheel, surf and ariel detergents respectively. About 20% inhibition was found with rin, tide, surf and ariel detergents where as enzyme retained its 90 and 100% activity in wheel, henko and ghadi detergent respectively at 1% concentrations. At 10% concentration enzyme retained 100% of its activity in henko and 90% activity in presence of rin, ghadi, wheel and surf detergents respectively, while 20% inhibition was found with tide and ariel detergents.

Summary

The present work was aimed to identify and characterize the serine proteases which having various industrial important properties from midgut of *P. americana*. Initially we screened the whole midgut extract against several detergents and organic compounds to identify the stable protease. We found that most of the *P. americana* midgut proteases showed stability to detergents and organic compounds. Among these identified stable proteases, highest activity isoforms were selected for further studies. Two *P. americana* midgut protease isoforms were purified to their homogeneity and characterized as trypsin and chymotrypsin on the basis of their activity and inhibition towards the specific substrates and inhibitors respectively. Stability of these enzymes towards surfactant, strong oxidizing agent, heavy metal ions, organic solvents and various commercial detergents have been checked. The purified proteases revealed excellent stability and compatibility towards detergents, oxidizing, reducing, and bleaching agents. In addition they also showed excellent stability towards organic solvents and commercial detergents. These properties of *P. americana* midgut proteases are useful to exploit them for commercial application in various industries. From the obtained results, insects seem to be excellent and alternative source of industrially important proteases in near future. The properties of the proteases found in the present investigation can also be exploited by using it as an active component in enzymic debriders. The insect proteases appear to be potentially useful as an additive in detergent, stain remover and other bio-formulations. From an industrial perspective, being able to rapidly identify such enzymes is also important. It is believed that our study is fruitful in identifying such enzymes which can be exploited commercially.

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